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METABOLISM OF HYDRAZINE

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TECHNICAL REVIEW AND APPROVAL

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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals, "Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

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In rats exposed to atmospheric hydrazine, the log of blood hydrazine concentration was found to bear a more or less linear relation to atmospheric hydrazine levels. At 20-25 mg hydrazine/M^3, blood concentrations approached 100 nmole/ml, and were about 2 nmole/ml at atmospheric concentrations of about 3 mg/M^3. During exposure, blood concentrations tended to rise rather quickly in the first hour, then oscillated slowly around a generally constant level throughout six hours of exposure.

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Blood concentration of hydrazine following a single 1 mmole/kg dose decreased with a first phase halftime of about 45 minutes followed by a slower decrease with halftime of 27 hours. Less than one percent of such doses was excreted in bile. In vitro reaction of 5 m molar hydrazine in whole blood proceeded at a rate of about 1.6×10^{-2} μmole/ml/minute or about 10% in 30 minutes. With higher concentrations turnover becomes more rapid and can exceed the hemoglobin subunit equivalence by many fold.

Extensive efforts to locate ^{15}N-labelled urinary metabolites that would account for remaining uninventoried hydrazine have not yet succeeded.

PREFACE

The research described in this report was conducted from 1 October 1979 to 30 September 1980 in the Department of Agricultural Chemistry, Oregon State University, Corvallis, Oregon, under Contract F 33615-79-C-0517, Project 2312, Task VI, Work Unit 16. The principal investigator was Dr. F.N. Dost. The contract monitor for the Air Force Aerospace Medical Research Laboratory was Dr. K.C. Back, Chief, Toxicology Branch, Toxic Hazards Division.

INTRODUCTION

The metabolic disposition of hydrazine has been under study for perhaps 25 years, and the fate of this rather simple molecule is still largely undefined. Some earlier studies of urinary excretion were concerned more with identification of metabolites than with quantitation (McKennis, 1955; McKennis and Weatherby, 1956; McKennis et al., 1959), and later quantitative studies did not account for derivatives (Dambrauskas and Cornish, 1964). All sought excretory products only in the urine. As a consequence, recoveries in various species were less than 50% of the administered material. Some tissue concentrations were also measured (Dambrauskas and Cornish, 1964). In our own earlier work, we followed excretion of hydrazine and a hydrolyzable derivative of hydrazine, finding about 50% of a single 1 mmole/kg/dose excreted in those forms in 48 hours. We also devised a method for measuring $^{15}N_2$ excretion by respiration following treatment with ^{15}N-hydrazine, and found that about 25% emerged in that form (Dost et al., 1979).

One major objective of the current research has been to locate the 25% of administered hydrazine not found in our previous studies. The tissue accumulation data of Dambrauskas and Cornish (1964) and our own preliminary work show that hydrazine itself rises to significant levels in tissues, then drops sharply. This behavior is reflected in blood and urine concentrations. Our strategy has been to seek the missing excretory products in urine, on the provisional assumption that the sharp decrease in tissue hydrazine, and subsequent increase in urinary hydrazine is characteristic of all metabolites. Once found in urine, we should be able to learn more about the nature of the metabolite and ways of extracting it from tissues and blood. Because we do not know the chemical nature of the missing material, we must first learn whether there is more ^{15}N in urine than is accountable as dimethylbenzaldehyde (DMBA) reactive material.

We have thus far been singularly unsuccessful in finding the missing metabolite in urine. There has been major difficulty in achieving reliable, quantitative recovery of ^{15}N-hydrazine itself from urine following conversion to $^{15}N_2$ and measurement of the gas by mass spectroscopy. This is important because when seeking an unknown derivative that may constitute up to 1/3 of urinary ^{15}N, we must be sure that all presently known metabolites (hydrazine and its hydrolyzable derivative) are fully recoverable by both wet chemical measurement and ^{15}N mass spectrometric methods. If there are substantial discrepancies in the inventory of known substances, we cannot reliably determine unknowns.

The present work also includes studies of the relation between atmospheric hydrazine concentration and hydrazine concentration in blood, and in vitro reaction of hydrazine in blood and in urine, using both unlabeled and ^{15}N-hydrazine.

Our conviction that a non-DMBA reactive hydrazine derivative exists in urine has been strengthened by findings that when ^{15}N-hydrazine was incubated in urine, DMBA reactive material decreased substantially but very little $^{15}N_2$ was found.

METHODS

Animal management, surgical preparation and sampling

Male Sprague-Dawley rats (Charles River Laboratories) were used throughout this project. Animals were obtained at about 200 grams and quarantined and examined by the Laboratory Animal Resource Center. They were then maintained in the animal facilities of the Department of Agricultural Chemistry until they reached a weight of about 280 gm, at which time any needed surgery was performed. OSU pelleted rations and water were available ad libitum.

Indwelling cannulas for blood sampling were emplaced in the posterior vena cava under anesthesia with sodium pentobarbital, with ether supplementation as needed. All surgery was completed at least five days prior to experimental use.

Blood samples were usually 0.25 - 0.5 ml, depending on the expected concentration of hydrazine. At the lowest concentration, sample size was increased but sample numbers were decreased. A total sample volume limit of 2.0 ml was observed except when the animal was to be killed immediately.

Bile duct cannulas were installed about 18 hours prior to use and exteriorized through the lateral abdominal wall. Animals were permitted to recover from anesthesia and then placed in restraining cages. Hydrazine was injected through an i.p. cannula and bile was collected for 24 hours. Animals were euthanized with ether at the end of bile collection experiments.

Measurement of total non-volatile ^{15}N in urine

An adaptation of the Dumas procedure is used as described in Fiedler and Proksch (1975). Urine samples are subjected to acid hydrolysis to convert hydrolyzable derivatives to hydrazine, then reacted with alcoholic p-dimethylaminobenzaldehyde to prevent loss during drying. The samples are placed in combustion tubes on a rotary evaporator to remove ethanol and water. The drying process requires 24-36 hours of careful regulation to prevent sample loss. Copper oxide and calcium oxide are then placed in the ampoule which is sealed and reacted at 500°C for 24 hours. The ampoule is placed in the airlock of the high vacuum system and broken, gases are collected on activated carbon at liquid nitrogen temperature as described by Dost et al. (1979) and transferred to the mass spectrometer.

Measurement of hydrazine

Hydrazine was measured in blood and urine by a modification of the method of Reynolds and Thomas (1965) using p-dimethylaminobenzaldehyde (DMBA) as a color reagent. As used herein, the modified method is described in Dost et al. (1979).

Generation of hydrazine atmospheres, exposure of rats and blood sampling

Hydrazine vapor was generated by moving nitrogen gas through a sintered glass sparger into undiluted hydrazine in a cylindrical trap. Mixing surface area was increased by surrounding the sparger with glass beads. The hydrazine-saturated nitrogen was moved into an annular mixing chamber with room air moving at high speed through its center into the animal chamber (Fig. 1). The segment in which hydrazine was laden on nitrogen and the wall of the chamber for mixing with air were heated to about 35°C with resistance tapes. The rates of nitrogen flow through the hydrazine ranged up to 30 ml/min and total air flow was usually four liters/min.

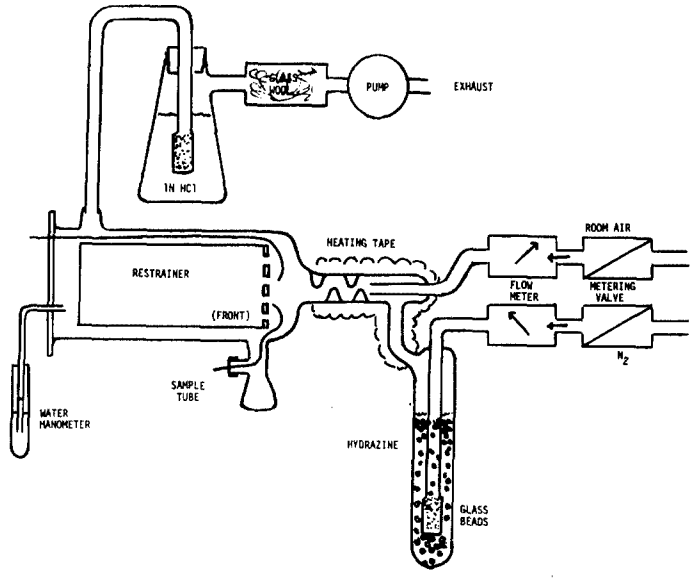

Figure 1. Chamber for exposure of rats to hydrazine atmosphere.

Hydrazine is quite reactive and also condenses very easily. Early attempts to create usable atmospheres were frustrated by highly variable measured chamber concentrations and often by complete disappearance of the atmosphere when an animal was present. Several mixing configurations were used and several heating patterns were tried with limited success. Any warming system that would also warm the subject was obviously unacceptable and it was finally recognized that in a workable apparatus we would have to accept at least limited aerosol formation. With that condition, it was decided that a very high total flow rate through the annular mixer with very quick distribution of hydrazine throughout the atmosphere would satisfy our needs. Some inconsistencies in chamber concentration at specific sampling points still occurred until we induced greater turbulence in the entry to the animal chamber.

Chamber atmospheres were initially measured by withdrawing 50 ml of vapor from sample probes placed at several points in the chamber. We found unacceptable inconsistencies that apparently arose through interaction between hydrazine and the sampling syringe. In the method now used, 200 ml of chamber atmosphere/minute is continuously withdrawn into a large acid trap throughout the duration of each experiment. The trap is then periodically sampled and the cumulative increase in concentration measured and plotted. If needed, any fraction up to total flow can be used. Chamber concentration may be continuously calculated as a function of the increase in trap concentration with time vs relative flow rate. Two typical plots of trap concentration are shown in Figure 2.

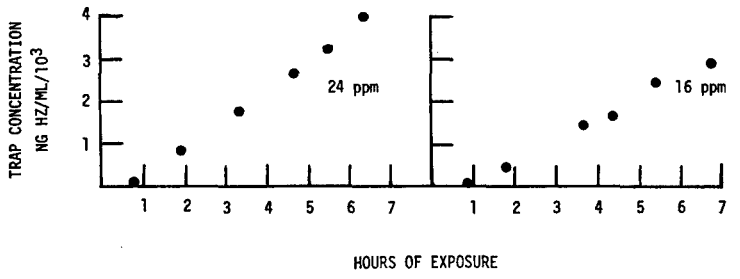

Figure 2. Typical patterns of downstream hydrazine trap concentrations from which exposure chamber concentrations were calculated.

In both the animal chamber and the measurement trap, flow rates are high enough that degradation or loss of hydrazine across the animal chamber or in sampling is negligible. The start-up procedure is to switch on the heating tape, establish the flow of nitrogen and room air at the desired rate, and run the system without a subject overnight for equilibration. In the morning, the animal is placed in the chamber, indwelling cannulas are connected, and trap collections are begun. The low flows of nitrogen are measured by a Hastings-Raydist LF 20 mass flowmeter, and air flow is measured by a Manostat flow meter (#36-541-03). Most experiments were continued for six hours with up to eight blood samples collected in some cases.

In theory, a desired concentration should be closely approached under preset conditions, but we find that it is not possible to simply "dial in" a specific concentration. There is intrinsic variability in the mixing components and apparently ambient conditions have considerable influence. Rough approximations to desired concentrations are possible, however, and once established, at set conditions a chamber concentration will remain stable throughout an experiment.

Hydrazine for reaction mixtures and analytical standards was freshly prepared as a 1.0 mM solution in glass distilled water.

Hemoglobin was measured by the cyanmethemoglobin procedure with Hycel reagents. Methemoglobin was determined by the method of Martin et al. (1960) in which the decrease in absorbance of hemoglobin solutions at 630 nm is measured after addition of NaCN.

RESULTS

A clear relationship was found between atmospheric hydrazine concentration and concentration of hydrazine in the blood of rats after six hours of exposure (Fig. 3).

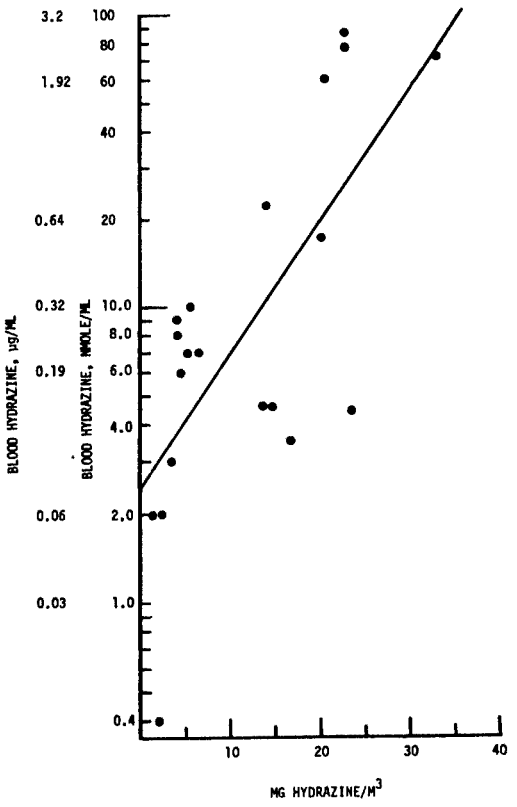

Figure 3. Relation between atmospheric hydrazine concentration and blood hydrazine concentration at the end of six hour exposures.

Over the usual six hour time course, blood hydrazine concentration oscillates moderately after the initial rise during the first hour of exposure. A series of six experiments is illustrated in Figure 4. In one case shown there was a continuing increase, but this pattern was seldom seen except at high chamber concentrations.

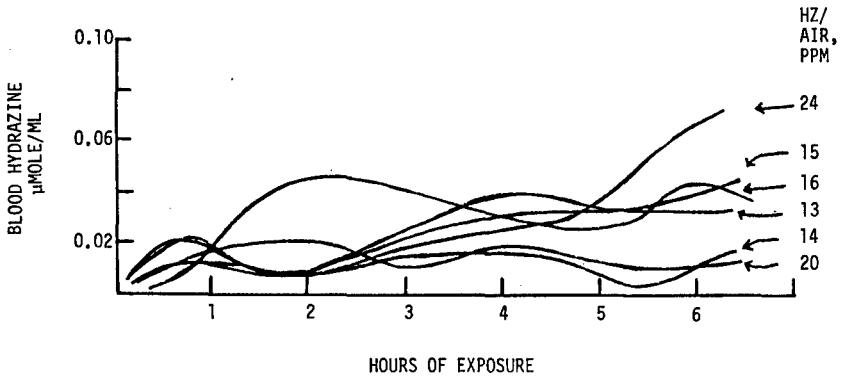

Figure 4. Typical time course of blood hydrazine concentration with time of exposure, at various atmospheric concentrations.

In earlier studies on this project and other research on metabolic effects of hydrazine, we have examined some aspects of low level hydrazine intoxication that can be related to the present data. These relationships are considered in the Discussion.

Behavior of blood hydrazine following a single i.p. injection

A previous report (Dost et al., 1979) contained a preliminary description of the behavior of hydrazine in blood following a single i.p. injection. That work has been refined and the curve in Figure 5 has been derived from three overlapping series of four animals each, covering most of the time course. The peak concentration occurred almost immediately, followed by a phase of rapid removal with a half time of 0.74 hours, followed by a slower phase with a half time calculated to be 26.9 hours. The acid hydrolyzable derivative did not reach a maximum level until about 30 minutes after injection, then declined rapidly.

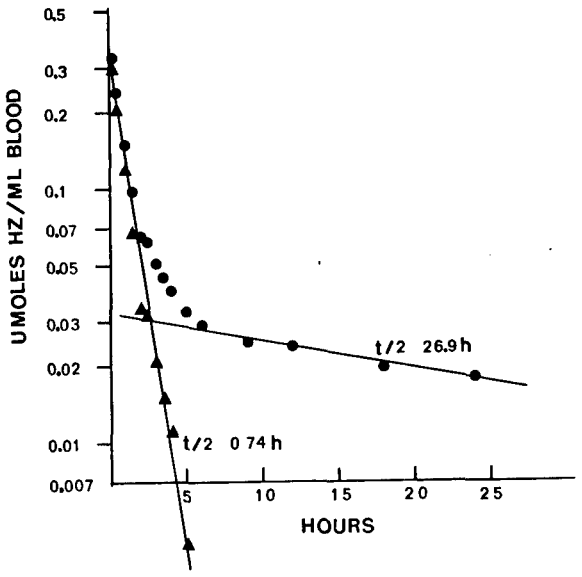

Figure 5. Time course of blood hydrazine concentration following a single dose of one mmole/kg i.p.

Corresponding urinary and respiratory excretion patterns of hydrazine and nitrogen gas derived from hydrazine can be found in a previous report (Dost et al., 1979).

Biliary excretion of hydrazine

Biliary excretion is apparently not an appreciable factor in disposition of hydrazine or its hydrolyzable derivatives. In each of the seven animals observed, less than one percent of administered hydrazine was found in the bile, in those forms, in 24 hours after administration.

Reaction of hydrazine with hemoglobin and whole blood

This is a series of exploratory experiments intended to provide direction for more detailed studies. Hydrazine was reacted at 37°C with whole blood, hemoglobin and methemoglobin solutions, and bovine serum albumin as a non-specific protein.

When hydrazine concentration in blood was high (25 mM; Hb = 13.1 gm/100 ml), the rate of hydrazine disappearance appeared to continuously decrease. The rate of removal of hydrazine at a beginning concentration of 5 mM was relatively slow and more or less linear (Fig. 6), averaging 1.6×10^{-2} μmoles/ml/minute, or between 9 and 10% in 30 minutes. (A concentration of 5 mM is quite high relative to concentrations expected in intoxication; death may occur at concentrations in vivo of 0.5 mM). These data are preliminary and are not sufficient for a suitable statistical analysis.

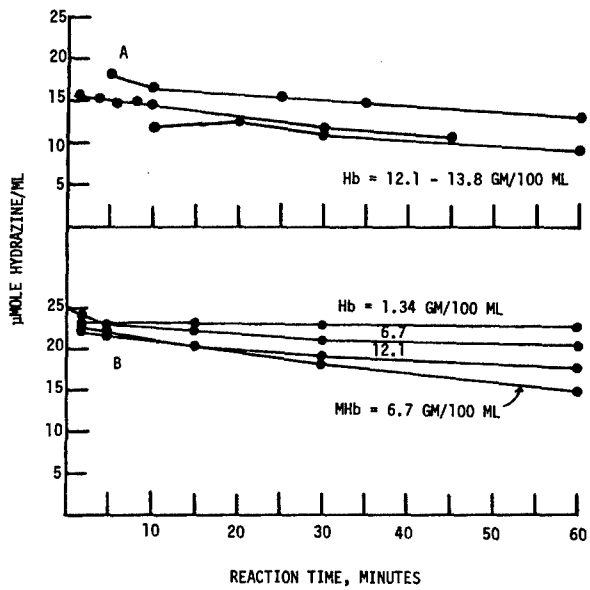

Figure 6. Reaction of hydrazine in vitro with, (A) whole blood, (B) hemoglobin in solution.

The data on reaction of hydrazine in cell-free hemoglobin solutions are quite limited as yet. At a hemoglobin concentration that is about 10% of physiological levels (1.34 gms/100 ml), the reaction rate of 5 mM hydrazine is essentially zero, but at physiological concentrations the rate appears to be about 1.5×10^{-2} μmoles/ml/min, essentially the same as that in whole blood. When the reaction was carried out with commercial hemoglobin (Sigma) (determined by us to be 100% methemoglobin), without exclusion of oxygen, the reaction proceeded somewhat faster than in a non-oxidized hemoglobin solution.

These latter findings must be amplified because 5 mM hydrazine induces up to 15% methemoglobin in vitro. If the reaction characteristics are about the same as reduced hemoglobin, no corrections are necessary, particularly at the early stages.

Positive controls with hydrazine in the presence of 6 gm bovine serum albumin/100 ml reacted at about 30% of the rate exhibited by whole blood preparations.

In rat plasma the disappearance rate was about 0.15×10^{-2} μmole/ml, about 10% of the rate in whole blood. The reaction in buffer alone was negligible.

In very preliminary observations it appears that most or all of the disappearance of hydrazine from blood in vitro can be accounted for as nitrogen production. Factors such as concentration dependence, oxygen saturation, and temperature have not been examined.

DISCUSSION

The possibility of a hydrazine derivative in urine that is not detectable by conventional methods still remains, and the apparent relative absence of hydrazine in bile does not preclude presence of a more complex and stable form in that fluid. Our supposition that the missing material is in the form of a stable complex of some kind is based on several considerations. We believe the missing hydrazine is not sequestered in tissues to any significant degree. Detectable tissue hydrazine has essentially disappeared by the end of the time courses. It does not seem reasonable that 25% of a dose of 0.5 LD_{50} should remain bound in tissues, particularly since an animal seems able to tolerate such doses day after day.

We do not think that the metabolite is in the form of single nitrogen metabolites or that it has disappeared into the amino nitrogen pool. We have been able to find almost no ^{15}N in ammonia, and consultations with other biochemists suggest that any amino nitrogen formation should result in some distribution into the ammonia pool. Other than absence in ammonia, however, we have yet to demonstrate absence of hydrazine nitrogen in amino acids; it is questionable whether the present level of sensitivity would sort out the very small amount of hydrazine nitrogen that would move into such a pool.

We have also reexamined the behavior of labeled hydrazine in urine. Questions had arisen about the quality of our evidence that $^{15}N_2$ in the animal chamber arose almost entirely from respiratory output, because we found that by chemical analysis as much as 70-80% of urinary hydrazine disappears at 25°C in three hours. We have found that less than 2% of that fraction emerges as nitrogen gas, however. We must conclude that it still exists in the urine, but is clearly not accessible to our analysis. Similar studies with blood, while preliminary, indicate that about 70% of the DMBA reactive material that disappears in vitro is accountable as $^{15}N_2$.

Various people have speculated on the possible nature of a stable hydrazine derivative, but no demonstrations of a reasonable molecule have emerged. Our approach has been to determine whether ^{15}N in any form in excess of that accounted for by chemical analysis is present in urine. To make this determination reliably, we must be able to recover ^{15}N-hydrazine and its known derivatives from urine quantitatively. We have had great difficulty in refining that determination, unlike our measurement of nitrogen gas. Until we can bring our variability down to 10% or less, there is little chance that we can demonstrate a 10-20% excess of ^{15}N over hydrazine nitrogen measurable by other means. We are also working with ways to oxidize all labile hydrazine in the urine sample, leaving only stable derivatives, but again there are limits to the precision of such a process. We are optimistic that our recent findings that urinary hydrazine may form a stable derivative in vitro may aid us in this search.

At this point we have accomplished a more satisfactory terminal inventory that has previously been possible, but there are a number of important questions yet to answer. Among these are, of course, the whereabouts and identity of the remaining terminal metabolite(s). The kinetics and specificity of tissue retention and release, both at the cellular and subcellular level, will be quite difficult to measure, partly because of the absence of full identity of products and the poor sensitivity of ^{15}N methodology. In view of the apparent carcinogenicity of hydrazine, it would be valuable to know whether it alkylates by way of derivatives or whether it acts as a local cell toxicant causing high rates of cell repair, with high nucleic acid turnover and increased probability of unrepaired mistakes in DNA synthesis.

A beginning has been made in relating exposure to blood levels and in turn to biochemical functions. This relationship is very rarely established for environmental or industrial intoxicants. We have shown previously that continuous intravenous infusion of hydrazine produces a relative steady state in blood hydrazine concentration that appears to be directly related to infusion rate (Dost et al., 1979). In comparing those findings with the present inhalation work, a relationship between infusion rate and atmospheric concentration can be drawn at a common blood hydrazine concentration. For example, an infusion of 83 μmoles hydrazine/kg/hour produces a blood hydrazine concentration of 0.05-0.07 μmole/ml. In our present work that blood level occurs at atmospheric hydrazine concentrations of about 0.6-0.7 nmole/ml (about 19-20 mg/m^3). If respiratory ventilation rate is assumed to be about 135-140 ml/minute for a 330 gm rat, with complete removal of hydrazine by the lung, we calculate that exposure

would be 0.7 nmole/ml x 140 ml/min = 98 nmole/min or 5.9 μmole/hour/0.333 kg or 17.7 μmole/kg/hr. This is about 20% of the amount that caused the same blood level when infused. On the basis of present data we cannot attribute this substantial difference to excretory or absorptive differences. The very rapid early conversion of infused hydrazine to N_2 is not sufficient to account for such a difference.

The possibility of an undetected metabolite in urine raises the possibility that such a substance can exist in blood. It is possible to speculate further that such a substance is biologically inactive, that both forms are generated during infusion and that only the active form arises in the lung.

We have not evaluated such biochemical functions as amine oxidase or DAO activity under inhalation of hydrazine. Until that is done we cannot expect to resolve the apparent discrepancy in blood levels between direct infusion and inhalation of hydrazine.

Plasma diamine oxidase activity in the rat can be stimulated by intravenous heparin. Hydrazine infused subcutaneously at 0.2 μmole/hour/rat (about 0.6 μmol/kg/hr) for four hours prior to heparin prevented the DAO increase. (A no-effect dose rate and duration were not established.)

If the apparently linear relationship between i.v. infusion rate and blood levels is extrapolated downward, an infusion of 0.6 μmole/kg/hr implies a blood level between 3 and 4 x 10^{-7}m, which is below our limit of reliable measurement. In turn, that blood concentration would suggest an equivalent atmospheric exposure that would probably be well below 0.1 mg/M^3. Infusion of as little as 0.04 μmole hydrazine/kg/hr caused measurable though not profound inhibition of plasma DAO. In vitro, we have found that 10^{-6}-10^{-7}M hydrazine blocks DAO activity. (Blood hydrazine concentrations of animals in atmospheres containing about 1 mg/hydrazine/M^3 are about 10^{-6}M.)

Oxidation in vivo of methylamine and putrescine is very sensitive to hydrazine. 2.5 μmole/kg/hr caused a discernible decrease in methylamine metabolism. An infusion of 2.5 μmole/kg/hr would produce a blood level of 1.5 nmol/ml which we predict would be produced by inhalation of about 0.03 to 0.04 mmole/L or 2-3 mg/M^3.

To the extent of the data available it would appear that prolonged exposure to atmospheres containing 0.1 mg hydrazine/M^3, or less, may produce measurable reversible biochemical change. In view of the discrepancy in the apparent exposures by inhalation and infusion it would be unwise to project one set of effects on the basis of the other.

The studies of in vitro reactions of hydrazine with blood and hemoglobin were intended to shed light on the possible association of $^{15}N_2$ production by intact animals with reaction in blood during the period of initial dispersal of the dose. The rates observed in these experiments indicate that interaction with blood components may make a limited contribution to, but is not the major source of, $^{15}N_2$ in vivo. In much earlier work we found that very high hydrazine concentrations in blood resulted in oxidation of hydrazine to the extent of many times the subunit equivalence of hemoglobin. It is clear with the present work, even though preliminary,

that as hydrazine concentration decreases, the turnover per hemoglobin subunit decreases. This suggests that little hydrazine should be directly reacted by hemoglobin at the concentrations encountered in non-lethal exposure.

The peculiar time course of $^{15}N_2$ production by rats has generated much speculation to account for the abrupt elaboration of substantial $^{15}N_2$, then reversion to a slow output over many hours. A question was raised about the assurance that considerable urinary hydrazine might have been converted to $^{15}N_2$ after it was voided. We tested this issue in a small system to assure greater sensitivity and found that hydrazine loss from urine was quite rapid, as measured by the DMBA method, but less than 2% of the loss could be accounted for as $^{15}N_2$.

This finding adds importance to our expectation that we should find a non-reactive metabolite in the urine. Whether it is formed in the bladder or only upon exposure to air cannot yet be speculated upon. It is unlikely that hydrazine and/or its reactive derivative(s) are altered while in the bladder, because the measurements of urinary constituents were remarkably consistent.

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